Paired-pulse facilitation and depression at unitary synapses in rat hippocampus: quantal fluctuation affects subsequent release

Dominique Debanne, Nathalie C. Guérineau, Beat H. Gähwiler and Scott M. Thompson

Brain Research Institute, University of Zurich, August Forel-Strasse 1, CH-8029, Zurich, Switzerland

1. Excitatory synaptic transmission between pairs of monosynaptically coupled pyramidal cells was examined in rat hippocampal slice cultures. Action potentials were elicited in single CA3 pyramidal cells impaled with microelectrodes and unitary excitatory postsynaptic currents (EPSCs) were recorded in whole-cell voltage-clamped CA1 or CA3 cells.

2. The amplitude of successive unitary EPSCs in response to single action potentials varied. The amplitude of EPSCs was altered by adenosine or changes in the $[Mg^{2+}]/[Ca^{2+}]$ ratio. We conclude that single action potentials triggered the release of multiple quanta of glutamate.

3. When two action potentials were elicited in the presynaptic cell, the amplitude of the second EPSC was inversely related to the amplitude of the first. Paired-pulse facilitation (PPF) was observed when the first EPSC was small, i.e. the second EPSC was larger than the first, whereas paired-pulse depression (PPD) was observed when the first EPSC was large.

4. The number of trials displaying PPD was greater when release probability was increased, and smaller when release probability was decreased.

5. PPD was not postsynaptically mediated because it was unaffected by decreasing ionic flux with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or receptor desensitization with aniracetam.

6. PPF was maximal at an interstimulus interval of 70 ms and recovered within 500 ms. Recovery from PPD occurred within 5 s.

7. We propose that multiple release sites are formed by the axon of a CA3 pyramidal cell and a single postsynaptic CA1 or CA3 cell. PPF is observed if the first action potential fails to release transmitter at most release sites. PPD is observed if the first action potential successfully triggers release at most release sites.

8. Our observations of PPF are consistent with the residual calcium hypothesis. We conclude that PPD results from a decrease in quantal content, perhaps due to short-term depletion of readily releasable vesicles.

Neurotransmitter is released when an action potential invades the axon terminal causing activation of voltage-dependent Ca^{2+} channels. The ensuing Ca^{2+} influx greatly increases the probability that vesicles docked at active sites in the axon terminal will fuse with the presynaptic membrane (Fatt & Katz, 1952; del Castillo & Katz, 1954*a*). Each vesicle contains a comparable amount of transmitter, so that the postsynaptic response produced by the release of a single vesicle, or *quantal response*, is relatively uniform (Fatt & Katz, 1952). At the neuromuscular junction, the number of vesicles released in response to seemingly identical presynaptic action potentials, i.e. the *quantal content*, varies randomly under conditions of constant release probability. On the other hand, at least some evidence suggests that release at the synapse formed by the Schaffer axon collateral of CA3 pyramidal cells with CA1 pyramidal cells may be only monoquantal (Rastaad, Storm & Andersen, 1992).

At the neuromuscular junction, there is a very large number of quanta available to respond to a presynaptic action potential, but each responds with a relatively low probability (del Castillo & Katz, 1954*a*). The probability

that one vesicle is released has been shown to be normally independent of the release of any other vesicle (Fatt & Katz, 1952; Barrett & Stevens, 1972). Nevertheless, when two nerve stimuli are applied in short succession to a mammalian neuromuscular junction, the amplitude of the second endplate potential is considerably reduced compared with the first (e.g. Thies, 1965; Betz, 1970), a phenomenon known as paired-pulse depression (PPD). When, on the other hand, the two stimuli are delivered under conditions in which the release probability has been reduced, such as after increasing the external $[Mg^{2+}]/[Ca^{2+}]$ ratio, the second response is larger than the first, and this process is known as paired-pulse facilitation (PPF; del Castillo & Katz, 1954*a*, *b*; Thies, 1965; Katz & Miledi, 1968). In the hippocampus, PPF of excitatory synaptic potentials in areas CA1 and CA3 is observed when large numbers of axons are stimulated (e.g. Creager, Dunwiddie & Lynch, 1980; Manabe, Wyllie, Perkel & Nicoll, 1993). Paired-pulse depression of excitatory synaptic transmission in areas CA1 and CA3 has, to our knowledge, only been described briefly (Storm, 1992).

Paired-pulse facilitation is traditionally accounted for by the residual calcium hypothesis of Katz & Miledi (1968). That is, a small fraction of the Ca^{2+} that enters the terminal in response to the first action potential remains for several hundred milliseconds. This amount of residual Ca^{2+} is too small to trigger much transmitter release itself, but nevertheless adds significantly to the Ca^{2+} entering the terminal during the second action potential. The increase in the probability of vesicle fusion produced by the second action potential is thus considerably greater than that achieved by the first. Paired-pulse depression at the neuromuscular junction is not accompanied by a decrease in the sensitivity of the endplate to acetylcholine (Otsuka, Endo & Nonomura, 1962; Thies, 1965), and therefore, possibly results from a decrease in evoked transmitter release.

In most studies of short-term synaptic plasticity in the hippocampus, extracellular stimulation is used to activate large numbers of axons. With this technique, however, variation in EPSC amplitudes resulting from fluctuations in quantal content cannot be measured because individual responses result from the release of transmitter from many presynaptic fibres. More recently, so-called minimal extracellular stimulation techniques have been used in an attempt to trigger release from only one axon (Hess, Kuhnt & Voronin, 1987; Storm, 1992). Unfortunately, however, failures of presynaptic transmitter release cannot be unambiguously distinguished from failures of the stimulus to trigger an action potential in the axon. We have therefore used recordings from pairs of monosynaptically connected pyramidal cells in areas CA1 and CA3 of rat hippocampal slice cultures to reinvestigate the cellular basis of paired-pulse facilitation and depression. Hippocampal slice cultures are a favourable preparation to study transmitter release and synaptic plasticity between individual cells because the probability of recording from monosynaptically coupled pairs of CA3 and CA1 pyramidal cells is much higher than in acutely prepared slice preparations, although their individual properties are comparable with those in *ex vivo* slices (Debanne, Guérineau, Gähwiler & Thompson, 1995).

We have addressed the following questions. Do single presynaptic action potentials trigger the release of more than one quantum of glutamate? If so, is there any influence of random fluctuations in quantal content on the responses to pairs of presynaptic action potentials? Finally, what mechanisms underlie such short-term synaptic plasticity?

METHODS

Slice culture preparation

Hippocampal slice cultures were prepared and maintained as described previously (Gähwiler, 1981). In brief, the hippocampi were dissected from five to seven-day-old rat pups that had been killed by decapitation, 400 μm-thick slices were cut with a tissue chopper and attached to glass coverslips with clotted chicken plasma. The coverslip and slice were placed in individually sealed test-tubes containing semi-synthetic medium and maintained on a roller drum in an incubator for 2–4 weeks. For electrophysiological recording, cultures were transferred to a chamber mounted on an inverted microscope and continuously superfused with warmed (29 °C) saline containing (mM): Na^+, 149; Cl^-, 149; K^+, 2·7; Ca^{2+}, 2·8; Mg^{2+}, 2·0; HCO_3^-, 11·6; $H_2PO_4^-$, 0·4; glucose, 5·6 and Phenol Red, 10 mg l^{-1}; pH 7·4. In some experiments the concentrations of Ca^{2+} and Mg^{2+} were altered as described in the text. All drugs were prepared as frozen, aqueous stock solutions, and applied by bath perfusion. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Tocris Cookson (Bristol, UK), aniracetam and 1,3-dipropyl-8-*p*-sulphophenylxanthine (DPSPX) were purchased from Research Biochemicals Int. (Natick, MA, USA), and adenosine was purchased from Sigma.

Electrophysiology

Presynaptic cells were impaled in stratum pyramidale using sharp microelectrodes filled with 1 M potassium methylsulphate and their membrane potential was amplified ×100 (Axoclamp-2A, Axon Instruments). Electrode resistance ranged from 40 to 60 MΩ. EPSCs were recorded from CA3 or CA1 pyramidal cells using whole-cell voltage clamp (Axopatch 200A, Axon Instruments). The patch pipette (2–5 MΩ) contained the following solution (mM): potassium gluconate, 140; Hepes, 5; EGTA, 1·1 and $MgCl_2$, 2; titrated to pH 7·2 with 0·5 N KOH. Eleven of the cell pairs were between a CA3 and a CA1 cell, and sixteen were between two CA3 cells and the data have been pooled except where indicated. Pyramidal cells were identified electrophysiologically, as described previously (Debanne *et al.* 1995). The criteria for establishing that EPSCs between cell pairs were monosynaptic included relatively short and invariant onset latencies and the ability to follow brief high frequency stimulus trains, as described in detail elsewhere (Debanne *et al.* 1995). Failures of transmission were judged subjectively for each trace. The integral of the mean 'failure' response, calculated for a 20 ms window centred at the peak of the EPSC, did not differ from an integral calculated for a 20 ms window preceding the presynaptic action potential, i.e. these responses could not be distinguished from the recording noise.

Pairs of action potentials were elicited in the presynaptic cell with short depolarizing current pulses (each 10–20 ms duration, 0·2–0·7 nA) separated by 20–3000 ms. Pairs of pulses were delivered at intervals of 7–10 s. The analog signals from the two electrodes (voltage and current) were digitized at 18 kHz and recorded on a video tape recorder. Off-line acquisition of 200 ms sequences was performed on an IBM PC with a digitization rate of 8–10 kHz (Acquis1, DIPSI Industrie, Asnières, France). Responses could be averaged by aligning the first presynaptic action potential using automatic peak detection. Only those responses in which the second action potentials were synchronized (>20) were included for calculation of the mean responses presented in Figs 3–6 and 8.

The inverse of the square of the coefficient of variation (CV^{-2}) was calculated as the square of the mean EPSC amplitude divided by its variance (see Faber & Korn, 1991). The results have been expressed as means ± standard error of the mean. Curves have been fitted to the data using Fig.P software (Biosoft, Cambridge, UK).

RESULTS

Single action potentials in CA3 pyramidal cells triggered EPSCs in monosynaptically coupled CA3 or CA1 pyramidal cells whose amplitude, but not latency, fluctuated (Fig. 1*C*) (Debanne *et al.* 1995). In all experiments, postsynaptic cells were whole-cell voltage clamped at a membrane potential of −60 mV, at which the EPSC is entirely mediated by so-called α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate

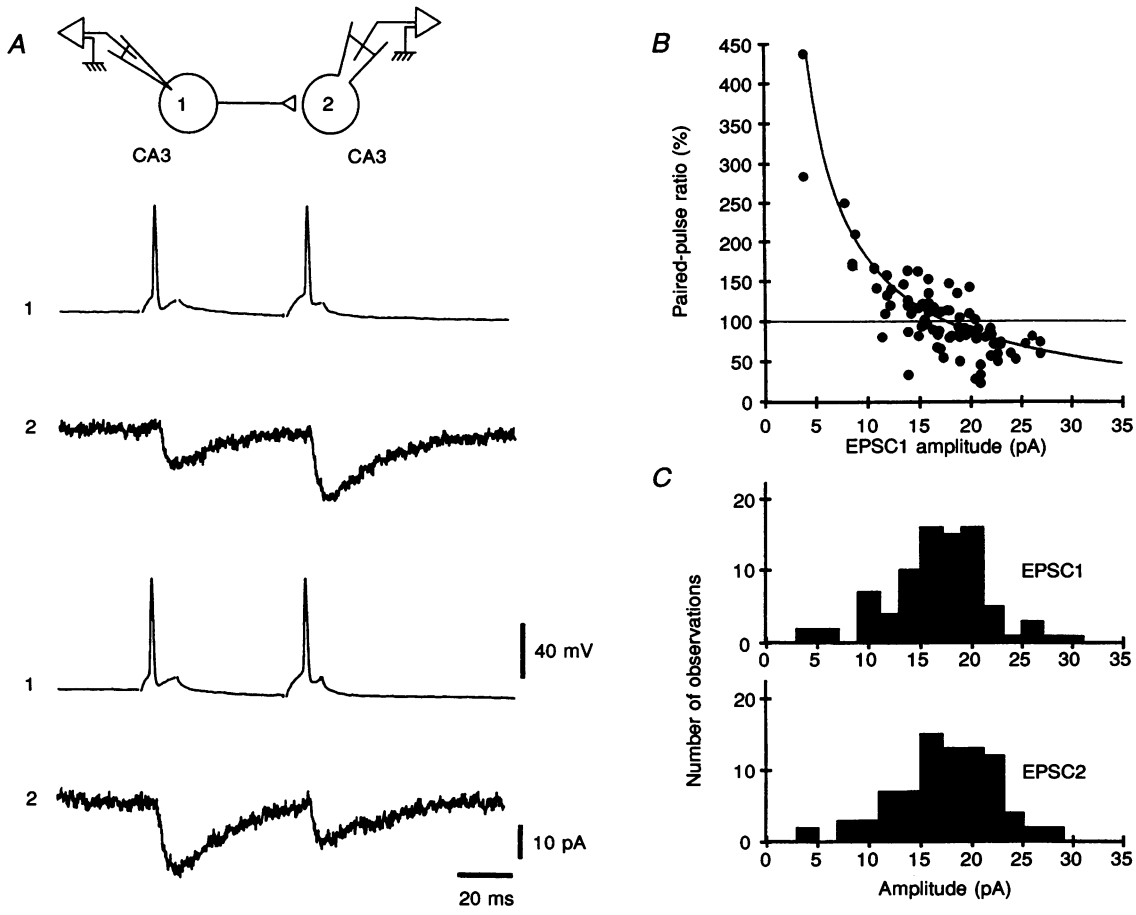

Figure 1. Paired-pulse facilitation occurs when EPSC1 is small and paired-pulse depression occurs when EPSC1 is large

A, pairs of CA3 pyramidal neurons were recorded simultaneously in hippocampal slice cultures. The presynaptic cell (1) was impaled with a sharp microelectrode and recorded in current-clamp mode and the postsynaptic cell (2) was voltage clamped at −60 mV using the whole-cell configuration of the patch-clamp technique. Pairs of action potentials were evoked every 7 s in cell 1 with an ISI of 50 ms. If the amplitude of the first EPSC (EPSC1) was small, then the amplitude of the second EPSC (EPSC2) was larger than EPSC1 (upper traces). If EPSC1 was large, then EPSC2 was smaller than EPSC1 (lower traces). *B*, plot of the paired-pulse ratio (EPSC2/EPSC1 × 100) as a function of the EPSC1 amplitude for the cell pair illustrated in *A*. Note that paired-pulse depression was always observed for the largest EPSC1s (>20 pA) and paired-pulse facilitation was always observed for the smallest EPSC1s (< 10 pA). This relation was well fitted by a hyperbolic function of the form: $y = (a/x) + b$. For this example, $a = 1860{\cdot}6$; $b = -5{\cdot}7$; ($r^2 = 0{\cdot}75$ where r is the correlation coefficient). *C*, histogram of EPSC1 and EPSC2 amplitudes for the cell pair illustrated in *A*.

(AMPA)/kainate-preferring receptors (Debanne *et al.* 1995). Under control conditions, failures of transmission were observed in response to < 10% of presynaptic action potentials (see below). Although we have not yet performed a detailed statistical analysis of the fluctuation of EPSC amplitudes, we shall assume for this paper that release at hippocampal synapses in slice cultures is fundamentally the same as has been described at the neuromuscular junction (Fatt & Katz, 1952; del Castillo & Katz, 1954*a*) and in acutely prepared slices (e.g. Malinow, 1991; Foster & McNaughton, 1991). That is, variations in EPSC amplitude result from the release of a randomly varying number of transmitter-containing vesicles, i.e. the quantal content of each EPSC varies. This assumption is justified by the results of several experimental manipulations described below.

Dependence of paired-pulse ratio on initial quantal content

Pairs of action potentials were elicited every 7–10 s in individual presynaptic CA3 pyramidal cells at intervals of 40–70 ms. The amplitude of the second EPSC (EPSC2) in a monosynaptically coupled CA3 or CA1 pyramidal cell was found to be dependent upon the amplitude of the first EPSC (EPSC1; Fig. 1*A*). The paired-pulse ratio (EPSC2/EPSC1 × 100) was plotted as a function of the amplitude of EPSC1. Paired-pulse facilitation occurred when the EPSC1 was of small amplitude whereas PPD was observed for large EPSC1 amplitudes (Fig. 1*B*). The distribution of these points could be well described by an hyperbolic function of the form $y = (a/x) + b$, and these curves were useful for comparing data before and after various experimental manipulations.

The dependence of the paired-pulse ratio on the amplitude of EPSC1 in individual trials was not random, but rather was determined mechanistically. EPSC2 was found to be 4·3 ± 0·8% greater ($P < 0{\cdot}0001$, Mann–Whitney U test, $n = 27$) than the mean of all EPSC2s, when EPSC1 was smaller than the mean of all EPSC1s. Conversely, EPSC2 was 3·7 ± 0·7% smaller ($P < 0{\cdot}0001$, Mann–Whitney U test, $n = 27$) than the mean of all EPSC2s, when EPSC1 was greater than the mean of all EPSC1s. If the fluctuation in the amplitude of EPSC1 results from changes in its quantal content, then we conclude that the amplitude of the response to the second action potential is inversely related to the quantal content of the first response.

The dependence of the paired-pulse ratio on the amplitude of EPSC1 was comparable for synapses formed by presynaptic CA3 pyramidal cells with both CA1 and other CA3 pyramidal cells. The paired-pulse ratios in the two types of pyramidal cell pairs were plotted as a function of the amplitude of EPSC1, after normalizing them to the largest individual response (Fig. 2). In both cases, PPD of as much as 50% was observed for the largest EPSC1s, whereas PPF of 300% was found for the smallest EPSC1s. The mean paired-pulse ratio for all responses was found to be 108 ± 6% for the CA3–CA3 pairs ($n = 16$), and 124 ± 14% for the CA3–CA1 pairs ($n = 11$), in control saline. Furthermore, PPD was observed in 47 ± 3% of individual trials in all pairs.

Effects of lowering release probability

The effects of two manipulations which decrease the probability of presynaptic glutamate release were tested on unitary paired-pulse responses. Paired-pulse facilitation of extracellularly evoked multifibre EPSCs in the hippocampus is enhanced when the external $[Mg^{2+}]/[Ca^{2+}]$ ratio is increased (e.g. Creager *et al.* 1980; Manabe *et al.* 1993). In our experiments, the amplitude of EPSC1 was found to be decreased by 63 ± 5% ($n = 10$) when the $[Mg^{2+}]/[Ca^{2+}]$

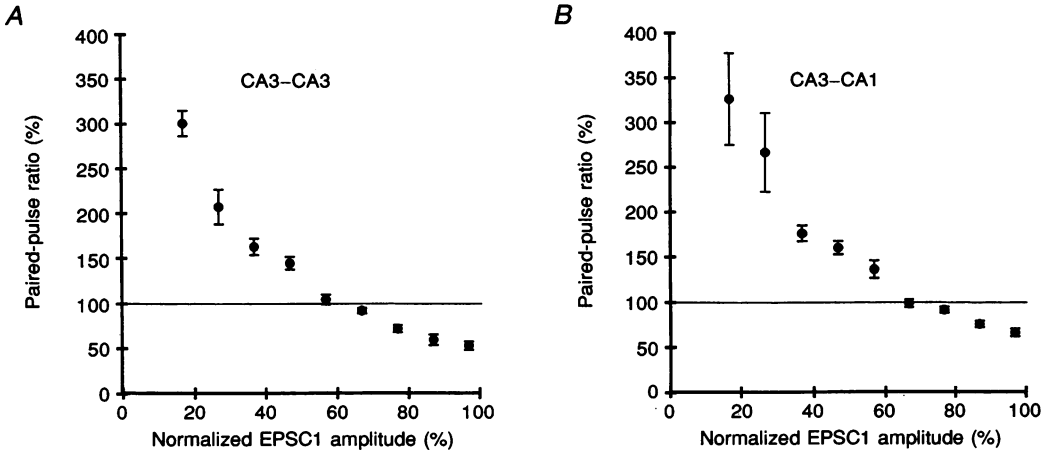

Figure 2. Summary of normalized paired-pulse ratios

The pooled paired-pulse ratios are shown for CA3–CA3 (*A*; $n = 7$) and CA3–CA1 (*B*; $n = 9$) pyramidal cell pairs. For each cell pair, EPSC amplitudes have been normalized to the amplitude of the largest event. Mean values of facilitation and depression were taken (± S.E.M.) in bins of 10%. At the synapses formed by CA3 cells with both target populations, PPF of up to 300% was found for the smallest events and PPD of up to 50% was observed for the largest events.

ratio was increased from 0·7 to 2·8 (Fig. 3). The inverse of the square of the coefficient of variation (CV^{-2}) of EPSC1 can be used as an indicator of the quantal content of a synaptic response, although many assumptions must be made (see Faber & Korn, 1991). The CV^{-2} of EPSC1 was calculated as described in Methods and was decreased by 89 ± 3% by this manipulation, consistent with an increase in its quantal content. In addition, the probability that presynaptic action potentials failed to trigger detectable transmitter release was much greater than in control saline (0% in control *vs.* 21 ± 4%, $n = 10$). In saline containing an increased $[Mg^{2+}]/[Ca^{2+}]$ ratio, the percentage of trials in which PPD was observed significantly decreased by 39 ± 7% ($n = 10$), and the mean paired-pulse ratio was increased by 43 ± 13% ($n = 10$) (Figs 3 and 7).

If the paired-pulse ratio is only dependent upon the amplitude of EPSC1, then the hyperbolic function describing their relationship should equally well describe the data in both conditions. When the graphs of paired-pulse ratio as a function of EPSC1 before and after increasing the $[Mg^{2+}]/[Ca^{2+}]$ ratio were compared, however, it was found that most points were below the curve derived from the control data after reducing release probability (see Discussion and Fig. 3).

The probability of presynaptic glutamate release can also be decreased by application of adenosine (Lupica, Proctor & Dunwiddie, 1992), which has previously been shown to increase the paired-pulse ratio of multifibre EPSPs (e.g. Dunwiddie & Haas, 1985). In cell pair recordings, bath application of low concentrations of adenosine (0·5–1 μM) decreased the amplitude of EPSC1 by 50 ± 8% ($n = 5$), decreased the CV^{-2} of EPSC1 by 66 ± 13% and increased the number of presynaptic action potentials that failed to trigger detectable transmitter release (1 ± 1% in control *vs.* 19 ± 8%, $n = 5$). In the presence of adenosine, the percentage of trials exhibiting PPD decreased by 39 ± 11% ($n = 5$) and the mean paired-pulse ratio increased by 48 ± 21% ($n = 5$; Fig. 7).

We conclude that decreasing release probability, either by raising the $[Mg^{2+}]/[Ca^{2+}]$ ratio or by applying adenosine,

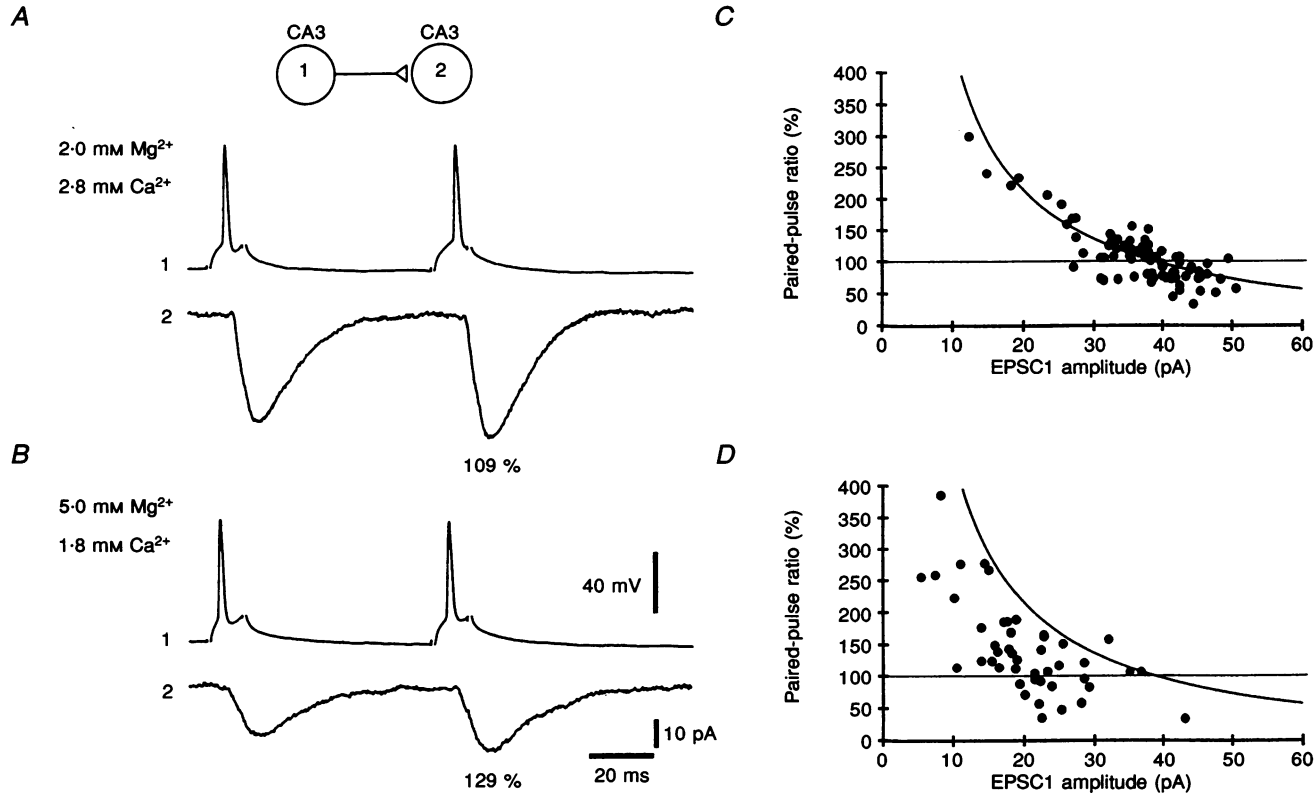

Figure 3. Effects of decreasing presynaptic release probability on paired-pulse ratio

The mean of all paired-pulse responses between two CA3 pyramidal cells in normal saline (external $[Mg^{2+}]$, 2 mM; $[Ca^{2+}]$, 2·8 mM) exhibited PPF (paired-pulse ratio of 109%; *A*). The paired-pulse ratio of each trial under these conditions is presented in *C* as a function of the EPSC1 amplitude. PPD was observed in 46% of the individual trials. The points on this graph were well fitted with a hyperbolic function ($a = 4767{\cdot}5$, $b = -123{\cdot}2$, $r^2 = 0{\cdot}74$). When release probability was decreased by raising external $[Mg^{2+}]$ to 5 mM and reducing external $[Ca^{2+}]$ to 1·8 mM, the mean of all trials in the same cell pair displayed larger PPF (paired-pulse ratio of 129%; *B*) and PPD was observed in only 26% of the individual trials (*D*). Note that the points fall below the line describing the data obtained for this cell pair under control conditions.

reduces the amplitude of EPSC1 by decreasing its quantal content, indicating that single action potentials in CA3 pyramidal cells result in the release of more than one quantum of transmitter onto postsynaptic pyramidal cells in control saline. Furthermore, decreasing the quantal content of EPSC1 by either method decreases the likelihood of observing PPD. These data provide additional evidence that the paired-pulse ratio is determined by the quantal content of EPSC1.

Effects of increasing release probability

The effects of experimentally increasing the probability of glutamate release were also tested on unitary paired-pulse responses. Decreasing the external $[Mg^{2+}]/[Ca^{2+}]$ ratio increases release probability and has been shown to decrease PPF of multifibre EPSPs in the hippocampus (Creager *et al.* 1980; Manabe *et al.* 1993). In our experiments, the mean amplitude of EPSC1 was increased by 47 ± 9% ($n = 12$; Fig. 4) and its CV^{-2} was increased by 165 ± 50% when the $[Mg^{2+}]/[Ca^{2+}]$ ratio was decreased from 0·7 to 0·13. Under these conditions, the mean paired-pulse ratio was decreased by 30 ± 5% ($n = 12$) and the probability of observing PPD in a given trial was increased by 81 ± 14% ($n = 12$; Figs 4 and 7).

As discussed above, the hyperbolic function describing the relationship between the paired-pulse ratio and the amplitude of EPSC1 should describe the data in both conditions equally well if the paired-pulse ratio is only dependent upon amplitude of EPSC1. When the graphs of paired-pulse ratio as a function of EPSC1 before and after increasing the release probability were compared, however, it was found that after decreasing the $[Mg^{2+}]/[Ca^{2+}]$ ratio most points were above the curve derived from the control data (see Discussion and Fig. 4). We conclude that increasing the quantal content of EPSC1 by elevating release probability increases the likelihood of observing PPD.

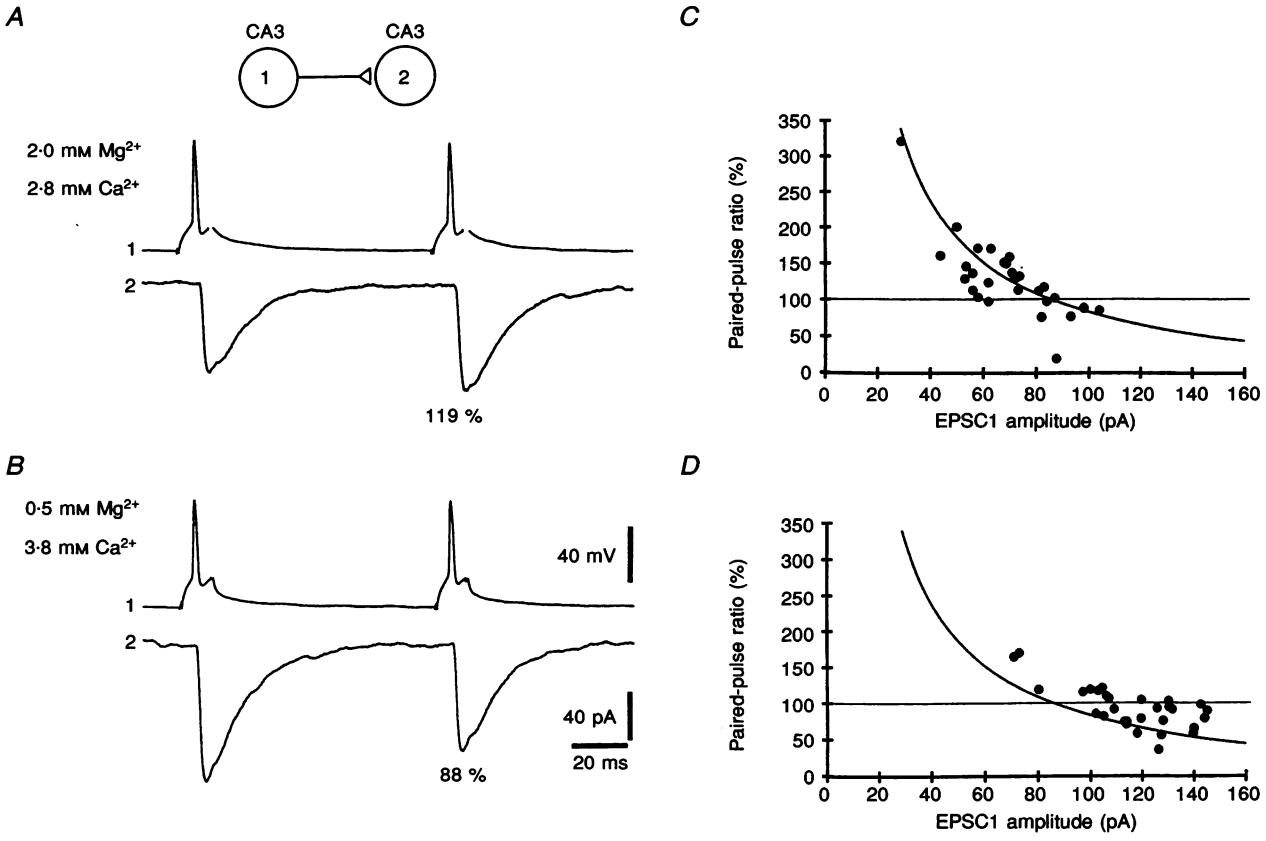

Figure 4. Effects of increasing presynaptic release probability on paired-pulse ratio

The mean of all paired-pulse responses between two CA3 pyramidal cells in normal saline (external $[Mg^{2+}]$, 2·0 mM; $[Ca^{2+}]$, 2·8 mM) exhibited PPF (paired-pulse ratio of 119%; *A*). The paired-pulse ratio of each trial under these conditions is presented in *C*, as a function of the EPSC1 amplitude. PPD was observed in 24% of the individual trials. The points in this graph were well fitted with a hyperbolic function ($a = 10408{\cdot}7$, $b = -121{\cdot}7$, $r^2 = 0{\cdot}71$). When release probability was increased by lowering external $[Mg^{2+}]$ to 0·5 mM and raising external $[Ca^{2+}]$ to 3·8 mM, the mean of all trials in the same cell pair displayed PPD (paired-pulse ratio of 88%; *B*) and PPD was observed in 67% of the individual trials (*D*). Note that the points fall above the line describing the data obtained for this cell pair under control conditions.

AMPA receptor desensitization is not responsible for PPD

AMPA receptors become desensitized during prolonged glutamate application. It is possible that glutamate released by the first action potential could leave the postsynaptic receptors desensitized and thus less responsive to the same amount of presynaptically released glutamate. We tested this hypothesis by using aniracetam, an inhibitor of AMPA receptor desensitization (Ito, Tanabe, Kohda & Sigiyama, 1990). Aniracetam (2 mM) increased the amplitude of EPSC1 by 38 ± 8·5% ($n = 5$; Fig. 5). Neither the mean paired-pulse ratio (32 ± 25·9% in control *vs.* 19·6 ± 10·6%, paired t test, $P > 0{\cdot}1$; Fig. 7) nor the probability of observing PPD in a given trial (38·4 ± 9% in control *vs.* 41·7 ± 3%, paired t test, $P > 0{\cdot}1$) were significantly changed by aniracetam, however. A comparison of the relationship between the paired-pulse ratio and the amplitude of EPSC1 revealed that there was an identical increase in the amplitude of EPSC2 for any given EPSC1 amplitude. We thus conclude that desensitization of postsynaptic AMPA receptors cannot account for the PPD observed when the quantal content of EPSC1 is large.

Endogenous adenosine is not responsible for PPD

Activity-dependent depression of the endplate potential at the neuromuscular junction of the frog has been attributed to the co-release of ATP with acetylcholine, degradation to adenosine and activation of presynaptic inhibitory receptors (Redman & Silinsky, 1994). Co-release of ATP or adenosine with glutamate could account for PPD in our experiments. This hypothesis was tested with the selective adenosine receptor antagonist DPSPX (5 μM) after increasing the probability of observing PPD by decreasing the $[Mg^{2+}]/[Ca^{2+}]$ ratio from 0·7 to 0·13. DPSPX produced no significant change in the paired-pulse ratio under these conditions, however (−4 ± 9%, $n = 4$). We conclude that endogenous production of adenosine is not involved in the generation of PPD at unitary synapses.

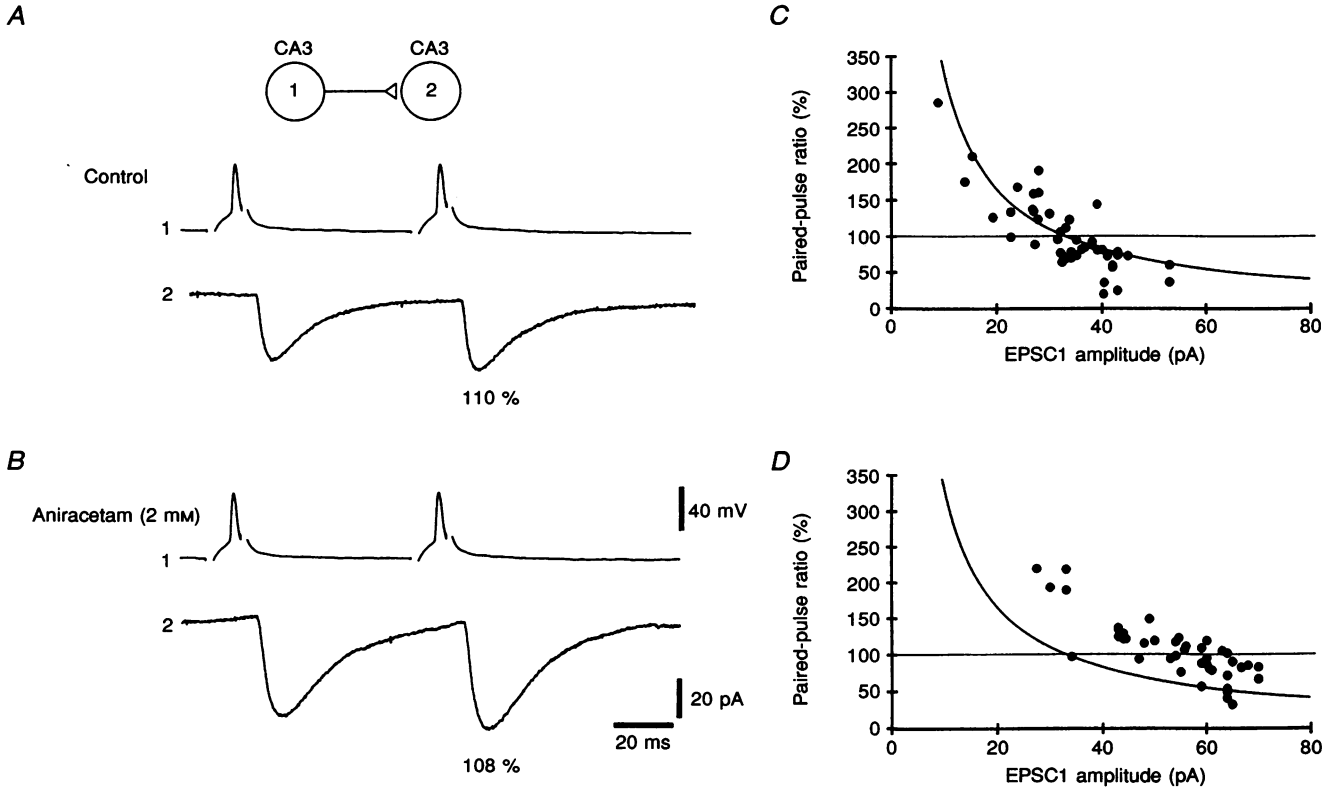

Figure 5. Increasing the EPSC amplitude with aniracetam does not affect PPD

In normal saline, the mean of all trials in a CA3–CA3 cell pair displayed PPF (*A*) and PPD was observed in 60% of the trials (*C*). The data from individual trials in this cell pair were well fitted with a hyperbolic function ($a = 3317{\cdot}6$, $b = -100{\cdot}7$, $r^2 = 0{\cdot}86$; *C*). Application of an inhibitor of AMPA receptor desensitization, aniracetam (2 mM; *B*), increased EPSC1 amplitude by 40%, but neither the paired-pulse ratio of the mean response nor the percentage of trials in which PPD was observed were significantly affected; *D*, PPD was observed in 53% of the individual trials. Note that the points all fall to the right of the line describing the data obtained for this cell pair under control conditions, consistent with an equal increase in the amplitude of both EPSCs.

A decrease in postsynaptic driving force is not responsible for PPD

At inhibitory synapses in the hippocampus, the depression observed after repetitive stimulation can be partially attributed to a use-dependent dissipation of Cl^- gradients (Thompson & Gähwiler, 1989). If a dissipation of postsynaptic ionic gradients in the dendritic spine occurred after EPSC1, then the amplitude of EPSC2 would decrease, even if presynaptic glutamate release was unchanged. In order to test this hypothesis, postsynaptic ionic flux was reduced by applying low concentrations (1 μM) of the AMPA/kainate receptor antagonist CNQX. After first increasing the probability of observing PPD by decreasing the $[Mg^{2+}]/[Ca^{2+}]$ ratio, CNQX was found to decrease the mean amplitude of EPSC1 by $76 \pm 2\%$ ($n = 5$; Fig. 6). Nevertheless, the mean paired-pulse ratio was unchanged ($-4 \pm 6\%$ of control, $n = 5$; Figs 6 and 7). We thus conclude, that a decrease in postsynaptic ionic gradients cannot account for the PPD observed, when the quantal content of EPSC1 is large.

Taken together, these results lead us to conclude that PPD results from a decrease in presynaptic glutamate release in response to the second action potential.

Time course of PPF and PPD

Since changes in release probability influence the number of trials exhibiting PPF and PPD, we took advantage of these conditions to characterize the time course of each form of short-term plasticity in relative isolation. First, paired-pulse responses were obtained for several different interstimulus intervals under conditions in which release probability was low ($[Mg^{2+}]/[Ca^{2+}]$ ratio at 2·8), favouring the occurrence of PPF. As transmitter release in response to the first presynaptic action potential will affect the

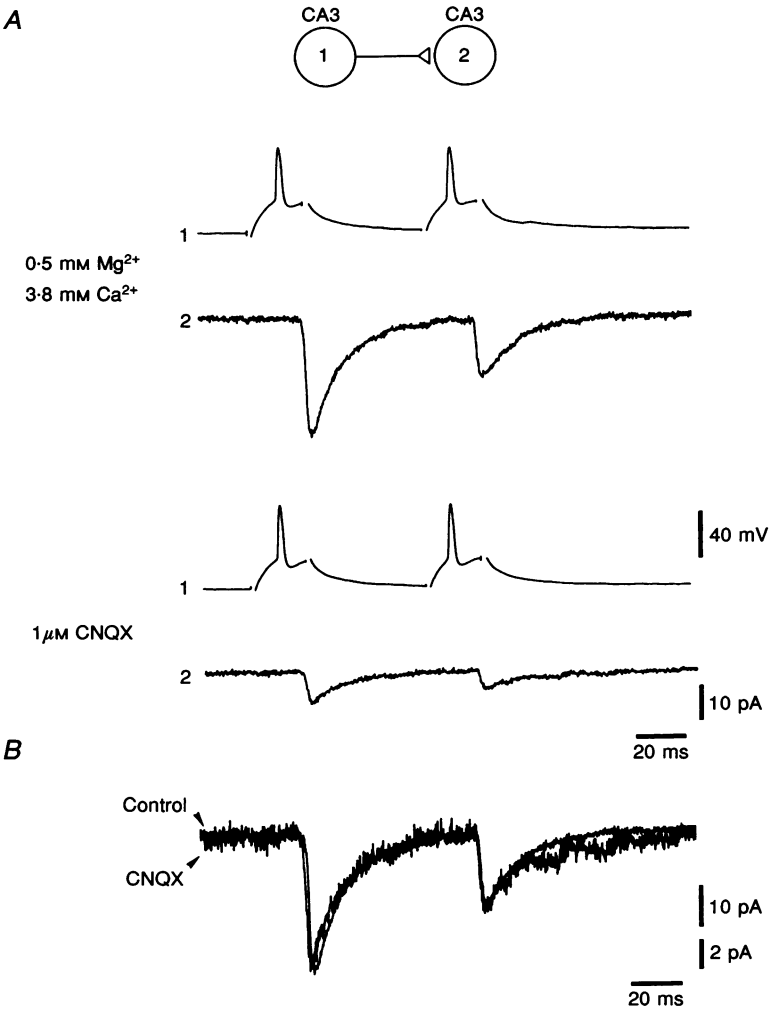

Figure 6. Decreasing the EPSC amplitude with CNQX does not affect PPD

A, in saline with a low $[Mg^{2+}]/[Ca^{2+}]$ ratio, the mean of all trials in a CA3–CA3 cell pair displays PPD (upper traces). Application of a low concentration of the AMPA receptor antagonist CNQX (1 μM) decreased the EPSC1 amplitude by 70% but did not affect the mean paired-pulse ratio (lower traces), as can be seen in the traces scaled to EPSC1 (*B*).

amplitude of the second response, PPF can be measured in the absence of concomitant PPD only when EPSC1 fails to trigger release (Fig. 8*A*). Such failures are relatively common under these experimental conditions. PPF was calculated as the ratio of the mean EPSC2 when the first action potential failed to trigger release to the mean of all EPSC1s. The paired-pulse ratio calculated in this way was found to be greater than the paired-pulse ratio calculated from the mean of all responses, in every cell pair and at all interstimulus intervals (ISI) < 1 s. The mean paired-pulse ratios for eleven CA3–CA3 pyramidal cell pairs are plotted as a function of the ISI in Fig. 8*B*. Surprisingly, PPF was found to be less for ISIs < 50 ms than for an ISI of 70 ms. No significant difference in the mean amplitude of the two EPSCs was observed when the ISI was ⩾500 ms. As the time course of PPF was calculated with a $[Mg^{2+}]/[Ca^{2+}]$ ratio of 2·8, however, it is likely that PPF has a larger amplitude and lasts longer in control saline.

Paired-pulse depression, on the other hand, is most readily obtained under conditions in which the release probability is elevated by decreasing the $[Mg^{2+}]/[Ca^{2+}]$ ratio to 0·13. As PPD is greatest when the quantal content of EPSC1 is largest (Fig. 8*C*), we calculated the paired-pulse ratio for eleven CA3–CA3 pyramidal cell pairs using only the largest EPSC1s obtained at each ISI. PPD was observed with all ISIs from 50 to 3000 ms (Fig. 8*D*). PPD of EPSC2 in these experiments should have been somewhat masked by concomitant PPF mediated by residual Ca^{2+} from the first action potential. Indeed, the level of depression appeared slightly smaller than expected for ISIs from 50 to 300 ms, probably because these ISIs correspond to the peak of facilitation, as seen in Fig. 8*B*. Since the recovery from PPF is complete after approximately 500 ms (Fig. 8*B*), at least for a $[Mg^{2+}]/[Ca^{2+}]$ ratio of 2·8, we have estimated the rate of recovery from PPD by fitting an exponential function to the data for all ISIs ⩾500 ms. This analysis indicated that no PPD occurs at ISIs ⩾5 s and that recovery occurs with a rate constant of 980 ms. It should be noted that PPD calculated in this way is somewhat distorted by the concomitant occurrence of PPF and this effect would be even greater in saline with the control $[Mg^{2+}]/[Ca^{2+}]$ ratio.

DISCUSSION

Transmitter release at hippocampal excitatory synapses

We have found that transmitter release fluctuates randomly at excitatory synapses formed by single CA3 pyramidal cells with other CA3 and CA1 pyramidal cells in hippocampal slice cultures. The distribution of amplitudes of unitary EPSCs was affected by manipulations that modify release probability, such as by changing the $[Mg^{2+}]/[Ca^{2+}]$ ratio or by applying adenosine to activate presynaptic inhibitory receptors. Our evidence thus suggests that a single presynaptic action potential in a CA3 pyramidal cell can cause the release of more than one quantum of neurotransmitter at its synapses with other CA3 or CA1 pyramidal cells, and that the number of quanta released fluctuates from trial to trial. In contrast to some previous suggestions (Rastaad *et al.* 1992), most evidence indicates that unitary excitatory synaptic responses between hippocampal pyramidal cells in acute slices are also multiquantal (e.g. Foster & McNaughton, 1991; Malinow, 1991; Lupica *et al.* 1992; Arancio, Korn, Gulyás, Freund & Miles, 1994).

The number of synaptic contacts formed by two pyramidal cells in hippocampal slice cultures is not known. We have

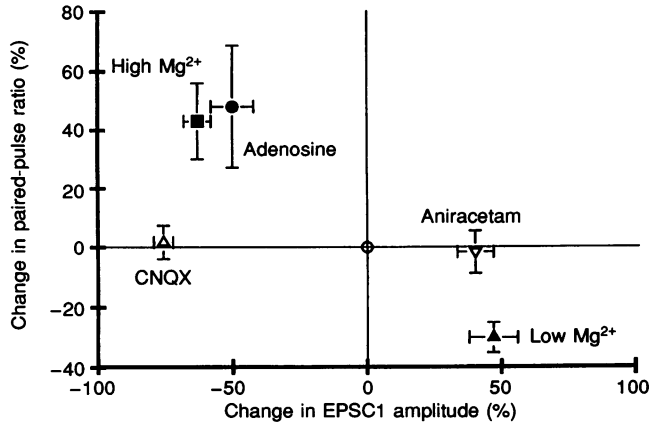

Figure 7. Summary of the effects of various experimental manipulations on the amplitude of EPSC1 and the mean paired-pulse ratio

Each symbol is the mean of all the experiments for that manipulation, including CNQX ($n = 5$), elevated $[Mg^{2+}]/[Ca^{2+}]$ ratio ($n = 10$), adenosine ($n = 5$), aniracetam ($n = 5$) and decreased $[Mg^{2+}]/[Ca^{2+}]$ ratio ($n = 12$). It can be seen that manipulations which alter postsynaptic glutamate sensitivity (CNQX and aniracetam) cause little or no change in the paired-pulse ratio whereas those manipulations that alter the presynaptic glutamate release (adenosine and changes in the $[Mg^{2+}]/[Ca^{2+}]$ ratio) strongly affect the paired-pulse ratio.

recently established (Debanne *et al.* 1995) that the amplitude of unitary synaptic responses between CA3 pyramidal cells in hippocampal slice cultures is comparable with that seen between CA3 cells in acutely prepared slices. In contrast to acute slices, where the amplitude of CA3–CA1 pyramidal cell unitary responses is 10-fold smaller than CA3–CA3 responses (e.g. Malinow, 1991), there is no difference in the amplitude of CA3–CA1 and CA3–CA3 responses in slice cultures. We have, therefore, suggested that the number of synaptic contacts in slice cultures is comparable with that *in situ*, but that numerous Schaffer collateral axons are severed during the preparation of acute slices (Debanne *et al.* 1995). CA3 pyramidal cells have been shown to form only one contact with inhibitory interneurons in *ex vivo* slices (Gulyás, Miles, Sik, Tóth, Tamamaki & Freund, 1993) and to elicit monoquantal EPSCs in these interneurons (Arancio *et al.* 1994). Although we have provided clear evidence for the release of multiple quanta in response to a single action potential, the data do not allow us to determine the number of sites from which they are released. We are currently attempting to perform a detailed quantal analysis of unitary synaptic currents and to label simultaneously both pre- and postsynaptic cells. In this way, we hope to be able to examine the relationship between the number of morphologically and physiologically determined release sites. We shall assume

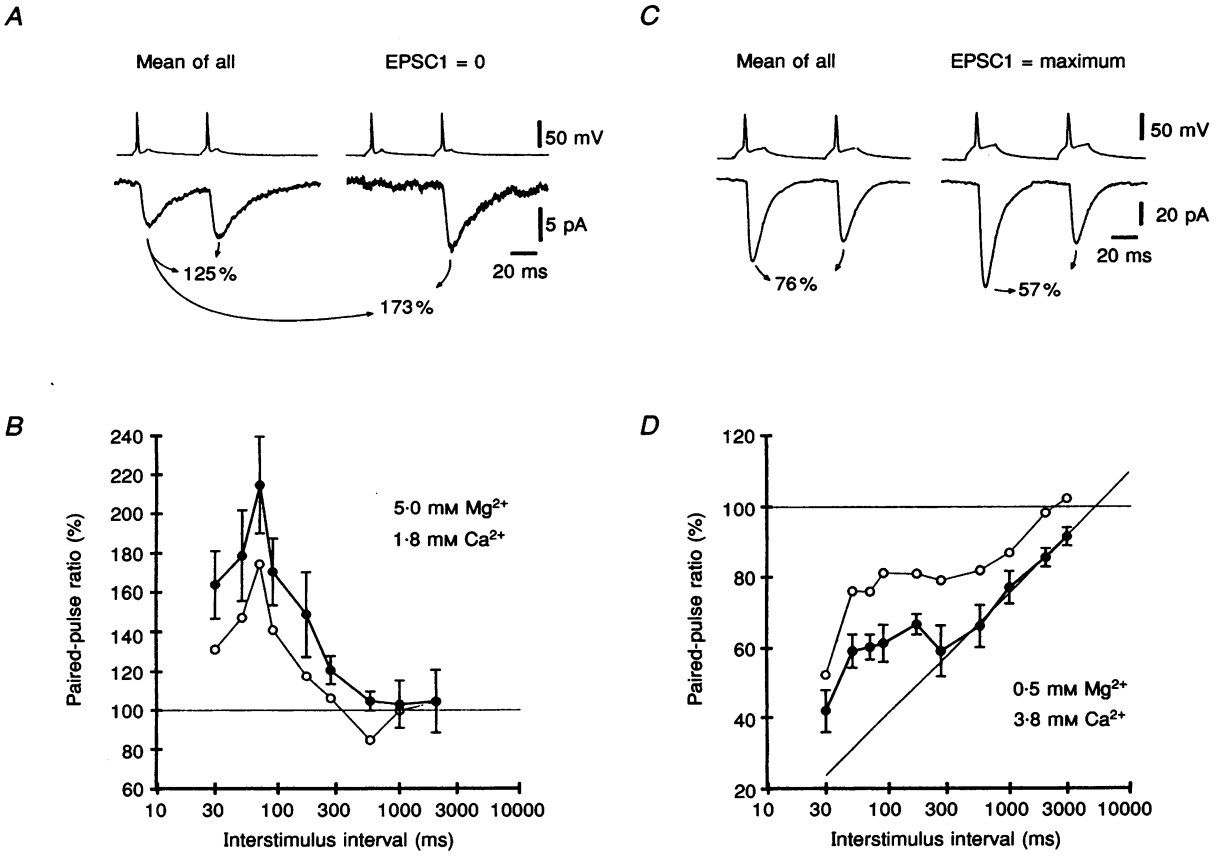

Figure 8. Time course of PPF and PPD

A, PPF is greater when the mean of all EPSC1s is compared with the mean of all EPSC2s preceded by a failure of EPSC1 (right) than to the mean of all EPSC2s (left; see text for explanation). *B*, plot of the paired-pulse ratio as a function of the interstimulus interval (ISI, logarithmic scale) in conditions of low glutamate release probability (external $[Mg^{2+}]$, 5 mM; $[Ca^{2+}]$, 1·8 mM). The paired-pulse ratio was first calculated using the mean of all EPSC1s and all EPSC2s (○). The effects of concomitant PPD were then corrected by using only EPSC2s which were preceded by a failure of transmission (●). PPF was then observed for all ISIs < 500 ms. *C*, PPD of EPSC2 is greater when it is preceded by the largest EPSC1s (right) than it is for the mean of all responses (left; see text for explanation). *D*, plot of the paired-pulse ratio as a function of ISI (logarithmic scale) in conditions of high glutamate release probability (external $[Mg^{2+}]$, 0·5 mM; $[Ca^{2+}]$ 3·8 mM). The paired-pulse ratio was first calculated using all trials (○) and then recalculated to minimize the contribution of concomitant PPF by using only the trials with the largest 10% of EPSC1s (●). PPD was then found to occur for all ISIs < 5 s. Note that PPD is weaker at ISIs of 30–300 ms, presumably because of some effects of simultaneous PPF. The data points from 500 ms to 3 s were well fitted ($r^2 = 0{\cdot}989$) with an exponential function of the form: $y = A \exp(-x/\tau) + k$, where $A = -46{\cdot}88$, $k = 92{\cdot}88$ and the rate constant $\tau = 980$ ms.

for simplicity that there are multiple release sites, each of which only releases a single quantum of transmitter in reponse to an action potential, as has been established at the inhibitory synapse of the goldfish Mauthner cell (Korn, Mallet, Triller & Faber, 1982). We shall also assume, for the remainder of the discussion, that release can be described by a compound binomial in which a single axon forms n release sites with the postsynaptic pyramidal cell, each with its own probability of release, P, which may or may not be uniform (see Redman, 1990).

We have observed that when pairs of action potentials are elicited in a single presynaptic cell, the amplitude of the second EPSC in a postsynaptic cell depends upon the amplitude of the first EPSC. More specifically, the paired-pulse ratio is a function of the quantal content of the first EPSC, and either PPF or PPD may be observed in individual trials under control conditions. The amplitude of EPSC1 is determined by the number of release sites at which transmitter is liberated. As discussed below, our data demonstrate that the probability of release at any given release site will be determined in part by its recent history. We suggest that PPF will be observed if the first action potential fails to release transmitter at most of the release sites formed by the axon of the presynaptic cell, whereas PPD will be observed if the first action potential successfully triggers transmitter release at most release sites.

Paired-pulse facilitation

When the quantal content of EPSC1 was relatively small, paired-pulse facilitation occured, i.e. EPSC2 had a larger amplitude than EPSC1. Such PPF did not require the successful release of transmitter in response to the first action potential, PPF was greatest after a failure of the first action potential to evoke transmitter release. It is probable that the increase in the amplitude of EPSC2 resulted from an increase in its quantal content, as previously reported (e.g. Foster & McNaughton, 1991).

Katz & Miledi (1968) have proposed that the second of two paired action potentials at the neuromuscular junction triggers release with a higher probability than the first action potential as a result of a small but lasting elevation of the intracellular $[Ca^{2+}]$ in the axon terminal. We have estimated the time course of PPF, in the absence of concomitant PPD, and find that it is complete within 500 ms of the first action potential when the $[Mg^{2+}]/[Ca^{2+}]$ ratio was 2·8. This time course presumably reflects the clearance of residual Ca^{2+} from the nerve terminal, and is consistent with previous estimates made in the hippocampus using Ca^{2+} indicator dyes (Hess & Kuhnt, 1992).

It was observed that the dependence of the paired-pulse ratio on the amplitude of EPSC1 was affected by changes in the $[Mg^{2+}]/[Ca^{2+}]$ ratio. For a given EPSC1 amplitude, i.e. quantal content, the paired-pulse ratio was consistently less than that seen in control conditions when the $[Mg^{2+}]/[Ca^{2+}]$ ratio was increased (Fig. 3) and greater than in control conditions when the $[Mg^{2+}]/[Ca^{2+}]$ ratio was decreased (Fig. 4). If PPF results from residual Ca^{2+} in the presynaptic nerve terminal, then one may conclude reasonably that there is less residual Ca^{2+} in saline containing an elevated $[Mg^{2+}]/[Ca^{2+}]$ ratio and more residual Ca^{2+} in saline containing a reduced $[Mg^{2+}]/[Ca^{2+}]$ ratio.

Although the evidence linking Ca^{2+} influx to the generation of PPF is overwhelming, there has been controversy about whether PPF results from residual Ca^{2+} itself, or rather from some Ca^{2+}-activated process. It has been found, for example, that some facilitatory processes at the neuromuscular junction are enhanced by substitution of Ba^{2+} or Sr^{2+} for Ca^{2+} (Zengel & Magelby, 1980). Furthermore, manipulations of the levels of expression of various presynaptic proteins affect PPF in the hippocampus (Rosahl *et al.* 1993; Chapman, Frenguelli, Smith, Chen & Silva, 1995). We have made two observations which bear on this issue. First, PPF was greater for an ISI of 70 ms than for shorter ISIs. One explanation for this effect may be that the absolute level of the intracellular $[Ca^{2+}]$ achieved by the second action potential is less for very short ISIs. Such an effect has not been seen in studies using Ca^{2+} indicator dyes, however (Hess & Kuhnt, 1992). Alternatively, this difference in the time of maximal PPF may reflect the delayed onset of some enzyme-mediated facilitatory process, such as activation of a Ca^{2+}-dependent protein kinase. Second, we have observed that PPF and PPD occur randomly at a given synapse under control conditions. Measurements of the paired-pulse ratio in experiments performed with extracellular stimulation in the hippocampus must, therefore, be interpreted with caution because the responses will reflect the mean of the simultaneous occurrence of PPF and PPD in different synapses. For example, the increase in the paired-pulse ratio observed by Rosahl *et al.* (1993) in area CA1 of mice lacking synapsin I may reflect either an enhancement in the processes underlying PPF or a depression of the processes underlying PPD. If fewer synapses undergo PPD, the mean paired-pulse ratio from many synapses will be increased.

Paired-pulse depression

When the quantal content of EPSC1 is high, paired-pulse depression occurs, i.e. the amplitude of the EPSC2 is smaller than that of EPSC1. Paired-pulse depression thus depends on release and is, therefore, a use-dependent form of synaptic plasticity. We conclude that this form of PPD results from a presynaptic decrease in quantal content and not a decrease in postsynaptic glutamate sensitivity. First, reduction of AMPA receptor desensitization with aniracetam had no effect on PPD. Second, reduction of postsynaptic ionic flux with low concentrations of CNQX did not affect PPD. Previous studies utilizing paired stimuli at the neuro-

muscular junction have not reported a similar form of PPD routinely, most probably because such studies are often performed with the release probability lowered by an increase in the $[Mg^{2+}]/[Ca^{2+}]$ ratio, so that individual quanta may be resolved. As pointed out by del Castillo & Katz (1954*b*), this makes it highly unlikely that a given terminal responds to both stimuli. However, under control physiological conditions in which very many terminals respond, PPD of the endplate potential is observed (Thies, 1965; Betz, 1970).

Paired-pulse depression of excitatory synaptic transmission in areas CA1 and CA3 has not previously been characterized, perhaps because responses from large numbers of axons are sampled in typical experiments, each of which has a different quantal content in response to a single stimulus. Supporting this suggestion, when mean responses of a single CA3–CA1 cell pair under control conditions are taken, PPF of roughly 25% is observed, comparable with typical values in acute hippocampal slices (Creager *et al.* 1980; Manabe *et al.* 1993). Two factors account for the fact that the mean response is facilitated. First, the PPF observed with low initial quantal contents is greater than the PPD observed with high initial quantal contents. Second, some release occurs, even when the initial quantal content is near its maximum value, i.e. the paired-pulse ratio was never observed to be $< 60\%$, probably because even the largest EPSCs are unlikely to represent successful release at every release site. Interestingly, PPD of inhibitory synaptic transmission is commonly observed in the hippocampus and at least part of this depression may be due to comparable mechanisms to those we describe here (Wilcox & Dichter, 1994).

When two stimuli are delivered in close succession, the probability of release is higher after the second action potential due to residual Ca^{2+} from the first action potential, regardless of the quantal content of the first EPSC (del Castillo & Katz, 1954*b*). In principle, this residual Ca^{2+} ought to result in an increase in release probability for all of the second action potentials. It might therefore be expected that PPF would be observed in all trials.

As we have observed, the amount of PPD at the neuromuscular junction is positively correlated with the quantal content of the first response (Thies, 1965; Mallart & Martin, 1968; Betz, 1970), suggesting that there is a decrease in the number of quanta available to be released by the second action potential, i.e. a depletion of presynaptic vesicles. It has been suggested that this depletion is limited to a subset of vesicles having a higher release probability, so that a given terminal is less likely to respond if there has been a successful fusion of a synaptic vesicle with the presynaptic membrane in response to the first action potential (see Zucker, 1973, 1989; Korn & Faber, 1987).

Formally, the number of quanta released in response to a single presynaptic action potential is given by the product of the number of release sites formed by the axon of the presynaptic cell with its postsynaptic target, n, which remains constant, and their individual release probabilities, P. Vesicles exist in different functional states within the nerve terminal, with some vesicles associated with cytoskeletal elements, some vesicles docked at active sites, and some vesicles undergoing endocytosis and refilling (Heuser, Reese, Dennis, Jan, Jan & Evans, 1979). Within the population of docked vesicles, it is likely that some are primed (e.g. Bittner & Holz, 1992) so that they are released more readily than others. Following the formulation of Zucker (1973), the release of transmitter at any given site will be determined by the product of two factors: P_*, the probability that the release site contains docked and primed vesicles, and P_r, the probability that an action potential triggers release of docked and primed vesicles. At all release sites, it is likely that P_* is very high or 1 (Korn & Faber, 1987), provided the stimulation frequency is less than 0·2 Hz (see below). The quantal content of EPSC1 will therefore primarily reflect P_r. For EPSC2, however, P_* will be decreased at sites in which docked and primed vesicles were secreted in response to the first action potential, whereas P_r will be elevated due to residual Ca^{2+} at all release sites. Paired-pulse depression must therefore result because release occurred successfully at the majority of release sites; that is, at those sites, where P_* has decreased more than P_r has increased, so that the product of P_* and P_r is less than it was for EPSC1 (see also Korn & Faber, 1987). We thus conclude that PPD represents a depletion of the finite number of vesicles in the immediately releasable or primed pool (see also Horrigan & Bookman, 1994).

We have observed that PPD has a duration of up to 5 s. We suggest that this time reflects the recovery of P_* or, in biological terms, the docking and re-priming of vesicles in the readily releasable pool. After excluding the influence of concomitant PPF as much as possible, we estimate that this is a first order process with a rate constant of roughly 1 s, as given by the data in Fig. 8*D*. When more prolonged, less physiological stimuli are used to trigger release, then the recovery from depression occurs 10–40 times more slowly (Liu & Tsien, 1995; Stevens & Tsujimoto, 1995), perhaps indicating that restoring the number of vesicles available for refilling the readily releasable vesicle pool is much slower than re-priming.

Although paired-pulse facilitation and depression can be observed in many preparations under various experimental conditions, it is striking and unexpected to find that such processes co-exist at the synapse formed between two cells under control conditions. The dependence of these forms of short-term synaptic plasticity on the quantal content of a preceding presynaptic action potential implies that transmitter release at a given synapse will be a function of its recent history. It has been noted that synapses with a

high degree of facilitation will transmit high frequencies of presynaptic discharge most faithfully, whereas synapses displaying depression will transmit low frequencies of presynaptic discharge better (Zucker, 1989). Changes in release probability, by favouring the proportions of synapses displaying PPF or PPD, will thus affect the information-processing properties of the hippocampus by influencing its filtering characteristics.

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